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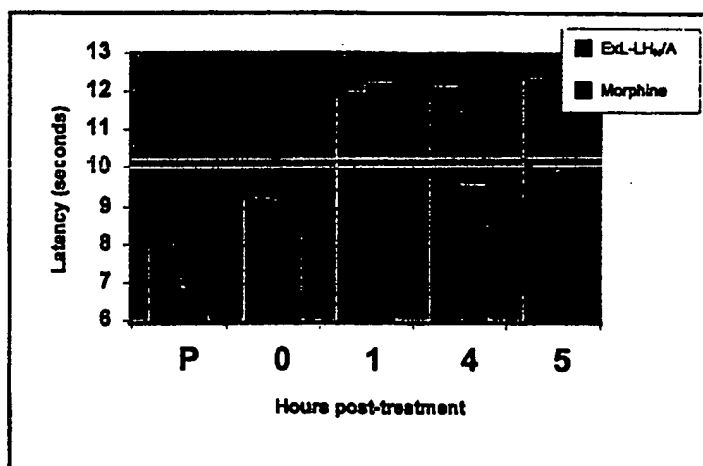
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(54) Title: CONJUGATES OF GALACTOSE-BINDING LECTINS AND CLOSTRIDIAL NEUROTOXINS AS ANALGESICS



(57) Abstract

A class of novel agents that are able to modify nociceptive afferent function is provided. The agents may inhibit the release of neurotransmitters from discrete populations of neurones and thereby reduce or preferably prevent the transmission of afferent pain signals from peripheral to central pain fibres. They comprise a galactose-binding lectin linked to a derivative of a clostridial neurotoxin. The derivative of the clostridial neurotoxin comprises the L-chain, or a fragment thereof, which includes the active proteolytic enzyme domain of the light (L) chain, linked to a molecule or domain with membrane translocating activity. The agents may be used in or as pharmaceuticals for the treatment of pain, particularly chronic pain.

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Conjugates of galactose-binding lectins and clostridial neurotoxins as analgesics.

Technical field

This invention relates to a class of novel agents that are
5 able to modify nociceptive afferent function. The agents
may inhibit the release of neurotransmitters from discrete
populations of neurones and thereby reduce or preferably
prevent the transmission of afferent pain signals from
peripheral to central pain fibres. The agent may be used
10 in or as a pharmaceutical for the treatment of pain,
particularly chronic pain.

Background

The sensation of pain due to injury or disease is carried
from the periphery to the brain by a multi-neuronal
15 pathway. The first part of this system comprises the
primary nociceptive afferents that form synapses with
secondary neurones in the dorsal horn of the spinal cord,
or the nuclei of the cranial nerves. These synapses pass
on the incoming information by the release of
20 neurotransmitters and neuromodulators such as glutamate
and substance P. These synapses are, therefore, possible
sites for intervention to alleviate pain, indeed one of
the modes of action of the opiate analgesics is to down-
modulate neurotransmitter release at these synapses.

25 Unfortunately, the opiates have a number of limitations as
drugs. Firstly, there are a number of chronic pain
conditions for which the opiates are not effective.
Secondly, the opiates have a number of side effects that
ar mediated both peripherally (constipation) and
30 centrally (respiratory depression and euphoria) which
present problems for long term use.

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There is, therefore, a need for the development of new pharmaceuticals for the treatment of pain, particularly chronic pain.

One approach to this problem is the use of new agents
5 containing fragments of clostridial neurotoxins
(WO96/33273).

The clostridial neurotoxins are proteins with molecular masses of the order of 150 kDa. They are produced by various species of bacterium of the genus *Clostridium*,
10 most importantly *C. tetani* and several strains of *C. botulinum*. There are at present eight different classes of the neurotoxins known: tetanus toxin, and botulinum neurotoxin in its serotypes A, B, C₁, D, E, F and G, and they all share similar structures and modes of action.
15 The clostridial neurotoxins are synthesised by the host bacterium as single polypeptides that are modified post-translationally to form two polypeptide chains joined together by a disulphide bond. The two chains are termed the heavy chain (H), which has a molecular mass of
20 approximately 100 kDa, and the light chain (L), which has a molecular mass of approximately 50 kDa.
Two distinct functions can be identified within the H-chain; binding and translocation. The carboxy-terminal half (H_C) is involved in the high affinity, neurospecific
25 binding of the toxin to cell surface acceptors, whilst the amino-terminal half (H_N) is central to the translocation of the toxin into the neuronal cell. For botulinum neurotoxin type A these domains are considered to reside within amino acid residues 872-1296 for the H_C, amino acid
30 residues 449-871 for the H_N and residues 1-448 for the LC. The minimal domains necessary for the activity of the light chain of clostridial toxins are described in J. Biol. Chem. Vol.267, No.21, July 1992, pages 14721-14729. The eight distinct neurotoxin light chains (L) are highly
35 specific zinc-dependent endopeptidases which each

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hydrolyse different but specific peptide bonds in one of three substrate proteins, synaptobrevin, syntaxin or SNAP-25. These substrates are important components of the neurosecretory machinery. The hydrolytic activity of the clostridial toxins results in a prolonged muscular paralysis. The functions of all three identified domains are necessary for the toxic activity of the clostridial endopeptidases.

Some of the clostridial endopeptidases, most notably botulinum neurotoxin type A, have been used as pharmaceutical agents for the treatment of a range of muscle dystonias. The flaccid paralysing action of the native botulinum toxins makes them appropriate for this use.

The use of fragments of clostridial neurotoxins for the desired purpose of analgesia is dependent on the invention of conjugates, or derivatives of these molecules, with a specific binding activity that will deliver the L-chain endopeptidase to the nociceptive afferent neurons in preference to other neurones in the relevant anatomical locus. Delivery of these conjugates includes binding to the cell surface, internalisation via an endosomal compartment and translocation of the clostridial endopeptidase activity into the cytosol.

Targeting of extracellular species to specific intracellular locations following endocytosis involves an appreciation of a number of possible targeting strategies. It is understood that early endosomes are part of the key sorting mechanisms of the cell, routing species to late endosome (and onto lysosomes for degradation), recycling to the cell surface or to the Trans-Golgi Network. Intracellular routing determinants have been suggested that determine the pathway and final destination of

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particular species (Mellman, 1996, Annu. Rev. Cell Biol., 12, 575-625).

Current data suggests that translocation of native clostridial neurotoxins occurs from an acidic
5 intracellular compartment, though the exact location and nature of the compartment is unknown (Montecucco & Schiavo, 1994, Mol. Micro. 13, 1-8). In patent WO96/33273 it is proposed that for an agent to be effective, the agent must target to an appropriate compartment for
10 translocation of the toxin. As an example of specific intracellular targeting, internalisation of the NGF-receptor is by specific endocytosis and retrograde routing (initiated by receptor-ligand complex), via acidic endosomes to the cell body, and an agent incorporating NGF
15 is given in support of WO96/33273.

Statement of Invention

The present invention relates to an agent that can reduce and preferably prevent the transmission of pain signals from the periphery to the central nervous system, thereby
20 alleviating the sensation of pain. Specifically, the invention can provide an agent that can reduce and preferably prevent the transmission of pain signals from nociceptive afferents to projection neurones. More specifically, the invention can provide an agent that can
25 inhibit the exocytosis of at least one neurotransmitter or neuromodulator substance from at least one category of nociceptive afferents.

In one aspect of the invention, an agent is provided which can be administered to the spinal cord, and which can
30 inhibit the release of at least one neurotransmitter or neuromodulator from the synaptic terminals of nociceptive afferents terminating in that region of the spinal cord.

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In a second aspect of the invention, there is provided an agent which can specifically target defined populations of afferent neurones, so that the effect of the agent is limited to that cell type.

- 5 In a third aspect of the invention, there is provided a method of treatment of pain that comprises administering an effective dose of the agent according to the invention.

In a fourth aspect of the invention, the agent can be expressed recombinantly as a fusion protein that includes
10 the required components of the agent.

Definitions

Without wishing to be limited by the definitions set down, it is intended in this description that the following terms have the following meanings:

- 15 Light chain means the smaller of the two polypeptide components of any of the clostridial neurotoxins. It is commonly referred to as the L-chain or simply L. An L-chain has a molecular mass of approximately 50 kDa, and it is a metalloprotease exhibiting high substrate specificity
20 for vesicle and/or plasma membrane associated proteins involved in the exocytotic process.

Heavy chain means the larger of the two polypeptide components of any of the clostridial neurotoxins. It is commonly referred to as H-chain or simply H and has a
25 molecular mass of approximately 100 kDa.

H_c fragment means a peptide derived from the H-chain of a clostridial neurotoxin which is responsible for binding of the native holotoxin to cell surface acceptor(s) involved in the intoxicating action of clostridial toxin prior to

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internalisation of the toxin into the cell. It may be approximately equivalent to the carboxy-terminal half of the H-chain, or the domain corresponding to that fragment in the intact H-chain.

5 H_N fragment means a fragment derived from the H-chain of a clostridial neurotoxin approximately equivalent to the amino-terminal half of the H-chain, or the domain corresponding to that fragment in the intact H-chain. It is characterised as:

10 A portion of the H-chain which enables translocation of that portion of the neurotoxin molecule such that a functional expression of light chain activity occurs within a target cell.

15 The domain responsible for translocation of the endopeptidase activity, following binding of neurotoxin to its specific cell surface receptor via the binding domain, into the target cell.

The domain responsible for formation of ion-permeable pores in lipid membranes under conditions of low pH.

20 The domain responsible for increasing the solubility of the entire polypeptide compared to the solubility of light chain alone.

LH_N means a fragment derived from a clostridial neurotoxin that contains the L-chain, or a functional fragment thereof, coupled to a H_N fragment.

BoNT/A means botulinum neurotoxin serotype A, and is a neurotoxin produced by *Clostridium botulinum*; it has a molecular mass of approximately 150kDa.

30 LH_N/A is LH_N that is derived from *Clostridium botulinum* neurotoxin type A.

Targeting Moiety (TM) means any chemical structure of an agent which functionally interacts with a binding site

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causing a physical association between the agent and the surface of a primary sensory afferent.

Primary sensory afferent is a nerve cell that can carry sensory information from the periphery towards the central nervous system.

Primary nociceptive afferent is a nerve cell that can carry sensory information from the periphery towards the central nervous system, where that information can result in a sensation of pain.

Lectin is any protein that binds to oligosaccharide structures.

Galactose-binding lectin is a lectin that binds to oligosaccharide structures in which the terminal residue is derived from galactose or N-acetylgalactosamine.

Detailed Description of the Invention

It can be seen from this disclosure that an agent for reducing or preventing the transmission of pain signals from peripheral, nociceptive afferent neurones to projection neurones has many potential applications in the reduction of the sensation of pain, particularly of severe chronic pain.

Lectins are a class of proteins, often glycoproteins, that bind to carbohydrate structures. Lectins are found across the whole range of life forms from viruses to mammals. The most commonly exploited sources are the abundant lectins found in the seeds of plants. Lectins have previously been labelled and used as cell surface markers.

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According to the invention, there is provided an agent that can inhibit the release of at least one neurotransmitter or neuromodulator or both from the synaptic terminals of nociceptive afferents.

5 It is known that such an agent can be produced based on the use of fragments of clostridial neurotoxin conjugated to a targeting ligand (WO96/33273). Given the known complexity of intracellular transport and the constraints on construct requirements, it is surprising that
10 conjugates between toxin fragments and a specific subclass of lectins that bind only to galactosyl residues form agents to produce analgesics that are particularly potent and selective. Inventions incorporating such lectins are the subject of this disclosure and several
15 examples are provided.

One example of a class of plant-derived, galactose-binding lectins are those that can be purified from the seeds of the genus *Erythrina*. These lectins have been characterised to exist predominantly as non-covalent
20 dimeric proteins with total molecular weights of approximately 60 kDa. Lectins have been isolated from several *Erythrina* species including: *E. corallodendron* (Gilboa-Garber and Mizrahi, 1981, Can. J. Biochem. 59, 315-320), *E. cristagalli* (Iglesias et al., 1982, Eur. J. Biochem. 123, 247-252), *E. indica* (Horejsi et al., 1980, Biochim. Biophys. Acta 623, 439-448), *E. arborescens*, *E. suberosa*, *E. lithosperma* (Bhattacharyya et al., 1981, Archiv. Biochem. Biophys. 211, 459-470) *E. caffra*, *E. flabelliformis*, *E. latissima*, *E. lysistemon*, *E. humeana*,
25 *E. perrieri*, *E. stricta*, and *E. zeyheri* (Lis et al., 1985, Phytochem. 24, 2803-2809).
30

These lectins have been analysed for their selectivity for saccharide binding (see e.g. Kaladas et al., 1982, Archiv. Biochem. Biophys. 217, 624-637). They have been found to

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bind preferentially to oligosaccharides with a terminal β -D-galactosyl residue.

A second example of a plant-derived, galactose-binding lectin with the desired binding specificity can be
5 obtained from *Glycine max* (soy) beans. This lectin (soya bean agglutinin, SBA) is a tetrameric protein with a total molecular weight of approximately 110 kDa. It binds to oligosaccharides containing galactose or N-acetylgalactosamine residues.

10 An example of a galactose-binding lectin from bacteria is PA-I, obtained from *Pseudomonas aeruginosa*. PA-I is a D-galactosephilic lectin with a molecular weight of about 13 kDa and it binds to galactose-containing oligosaccharides (Gilboa-Garber and Mizrahi, 1981, Can. J. Biochem. 59,
15 315-320).

These and other lectins of the sub-class of galactose-binding lectins can be used as targeting moieties (TM) for conjugates of the type described in WO96/33273. The requirements for TMs in these agents are that they show
20 specificity for the primary sensory afferents over other spinal nerves and that they lead to the internalisation of the agents into an appropriate intracellular compartment. The lectins of this invention fulfil these criteria. Surprisingly, in comparison to other lectins of
25 WO96/33273, they can fulfil these criteria more efficiently and can provide agents with enhanced selectivity for nociceptive afferent neurosecretion.

Thus, in one embodiment of the invention a galactose-binding lectin is conjugated, using linkages that may
30 include one or more spacer regions, to a derivative of the clostridial neurotoxins.

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- In another embodiment of the invention the agent is expressed in a recombinant form as a fusion protein. The fusion protein may be derived from nucleic acid encoding an appropriate fragment of a galactose-binding lectin, in addition to any desired spacer domains, with nucleic acid encoding all or part of a polypeptide of one serotype of neurotoxin. Such a nucleic acid may be a chimera derived from the nucleic acid encoding polypeptides from more than one serotype.
- 10 In another embodiment of the invention the required LH_N , which may be a hybrid of an L and H_N from different clostridial toxin serotypes, is expressed as a recombinant fusion protein with the galactose-binding lectin, and may also include one or more spacer regions.
- 15 In a further embodiment of the invention the required TM, L or LH_N and translocation domain components may be separately expressed in a recombinant form and subsequently linked, covalently or non-covalently, to provide the desired agent.
- 20 In a further embodiment of the invention the required translocation domain may be of a non-clostridial origin, comprising instead a peptide or other entity capable of similar or enhanced function. Examples would include, but not be restricted to, the translocation domain of
- 25 diphtheria toxin (O'Keefe et al., Proc. Natl. Acad. Sci. USA (1992) **89**, 6202-6206 ; Silverman et al., J. Biol. Chem. (1993) **269**, 22524-22532), the translocation domain of *Pseudomonas* exotoxin type A (Prior et al. Biochemistry (1992) **31**, 3555-3559), the translocation domains of
- 30 anthrax toxin (Blanke et al. Proc. Natl. Acad. Sci. USA (1996) **93**, 8437-8442) and a variety of fusogenic or hydrophobic peptides of translocating function (Plank et al. J. Biol. Chem. (1994) **269**, 12918-12924).

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Exploitation in Industry

The agent described in this invention can be used *in vivo*, either directly or as a pharmaceutically acceptable salt, for treatment of pain.

5 For example, an agent according to the invention can be administered by spinal injection (epidural or intrathecal) at the level of the spinal segment involved in the innervation of an affected organ for the treatment of pain. This is, for example, applicable in the treatment
10 of deep tissue pain, such as chronic malignant pain.

The present invention will now be described by reference to the following examples together with the Figures that show the following:

15 Figure 1. SDS-PAGE analysis of fractions from ExL-LH_N/A purification scheme

Figure 2. Cleavage of SNAP-25 by ExL-LH_N/A

Figure 3. SDS-PAGE analysis of fractions from EcL-LH_N/A purification scheme

20 Figure 4 SDS-PAGE analysis of fractions from SBA-LH_N/A purification scheme

Figure 5 Native gel analysis of ExL- and SBA-LH_N/A

Figure 6 Activity of ExL-LH_N/A on release of neurotransmitter from eDRG and eSC neurons

25 Figure 7 Activity of SBA-LH_N/A on release of neurotransmitter from eDRG and eSC neurons

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Figure 8 Activity of WGA-LH_N/A on release of neurotransmitter from eDRG and eSC neurons

Figure 9 Activity of ExL-LH_N/A in an *in vivo* electrophysiology model of analgesia

5 Figure 10 Activity of ExL-LH_N/A in an *in vivo* behavioural model of analgesia

Example 1. The Production of a conjugate between a lectin from *Erythrina cristagalli* and LH_N/A.

Materials

10 Lectin from *E. cristagalli* (ExL) was obtained from Sigma Ltd.

LH_N/A was prepared essentially by the method of Shone C.C., Hambleton, P., and Melling, J. 1987, *Eur. J. Biochem.* **167**, 175-180.

15 SPDP was from Pierce Chemical Co.

PD-10 desalting columns were from Pharmacia.

Dimethylsulphoxide (DMSO) was kept anhydrous by storage over a molecular sieve.

20 Denaturing sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using gels and reagents from Novex

Immobilised lactose-agarose was obtained from Sigma Ltd.

Additional reagents were obtained from Sigma Ltd.

Methods

25 The lyophilised lectin was rehydrated in phosphate buffered saline (PBS) to a final concentration of 10 mg/ml. Aliquots of this solution were stored at -20°C until use.

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The ExL was reacted with an equal concentration of SPDP by the addition of a 10 mM stock solution of SPDP in DMSO with mixing. After one hour at room temperature the reaction was terminated by desalting into PBS over a PD-10 column.

The thiopyridone leaving group was removed from the product by reduction with dithiothreitol (DTT, 5 mM, 30 min). The product of this reaction was analysed spectrophotometrically at 280 nm and 343 nm to determine the degree of derivatisation achieved. The degree of derivatisation achieved was 0.8 ± 0.06 mol/mol. The thiopyridone and DTT were removed by once again desalting into PBS over a PD-10 column.

The LH_N/A was desalted into PBSE (PBS containing 1 mM EDTA). The resulting solution (0.5-1.0 mg/ml) was reacted with a four- or five-fold molar excess of SPDP by addition of a 10 mM stock solution of SPDP in DMSO. After 3 h at room temperature the reaction was terminated by desalting over a PD-10 column into PBS.

A portion of the derivatised LH_N/A was removed from the solution and reduced with DTT (5 mM, 30 min). This sample was analysed spectrophotometrically at 280 nm and 343 nm to determine the degree of derivatisation. The degree of derivatisation achieved was 2.26 ± 0.10 mol/mol.

The bulk of the derivatised LH_N/A and the derivatised ExL were mixed in proportions such that the ExL was in greater than three-fold molar excess. The conjugation reaction was allowed to proceed for >16 h at 4°C.

The product mixture was centrifuged to clear any precipitate that had developed. The supernatant was concentrated by centrifugation through concentrators (with 10000-50000 molecular weight exclusion limit) prior to a two step purification strategy. As the first step, the concentrated material was applied to a Superose 12 column on an FPLC chromatography system (Pharmacia). The column

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was eluted with PBS and the elution profile followed at 280 nm.

Fractions were analysed by SDS-PAGE on 4-20% polyacrylamide gradient gels, followed by staining with
5 Coomassie Blue. The major band of conjugate has an apparent molecular mass of between 130-160 kDa; this is separated from the bulk of the remaining unconjugated LH_n/A and more completely from the unconjugated ExL. Fractions containing conjugate were pooled prior to the second
10 chromatography step; immobilised lactose-agarose. Selected post-Superose-12 fractions were applied to PBS-washed lactose-agarose and incubated for 2 hours at 4°C to facilitate binding. Lectin-containing proteins (i.e. ExL-LH_n/A conjugate) remained bound to the agarose during
15 subsequent washing with PBS to remove contaminants (predominantly unconjugated LH_n/A). ExL-LH_n/A conjugate was eluted from the column by the addition of 0.3M lactose (in PBS) and the elution profile followed at 280 nm. The fractions containing conjugate were pooled, dialysed
20 against PBS, and stored at 4°C until use.

In figure 1 is illustrated the SDS-PAGE profile during different stages in the conjugate purification scheme. Lanes 2 and 3 indicate ExL lectin and LH_n/A respectively prior to conjugation. Lanes 4, 5 & 6 represent conjugation
25 mixture, post-Superose-12 and post-lactose affinity chromatography samples respectively. Lane 6 is therefore indicative of the profile of the final conjugate material. Molecular weight markers are represented in lanes 1 & 7 with sizes indicated on the figure.

30 On the SDS-PAGE gel there are bands due to lectin alone in fractions containing the conjugate, this material is probably due to the non-covalent homo-dimeric nature of the ExL; where only one monomer of ExL is covalently attached to the LH_n/A the other is dissociated from the
35 complex by the SDS in the electrophoretic procedure giving rise to these bands. The absence of free lectin monomers

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was confirmed by native PAGE analysis and is illustrated in Figure 5. ExL-LH_n/A (lane 5) was analysed by non-denaturing PAGE. Samples were separated using 4-20% polyacrylamide gel for 6.75 hours, 125V, 4°C. The electrophoresis profile was compared to those of LH_n/A (lane 3) and ExL lectin only (lane 4). A range of marker proteins were analysed alongside; apoferritin (lane 6), β -amylase (lane 8), alcohol dehydrogenase (lane 7) and albumin (lane 9). Approximate molecular sizes are indicated.

Example 2. The production of a conjugate between a lectin from *Erythrina corallodendron* and LH_n/A.

The procedure for production of a conjugate between a lectin from *Erythrina corallodendron* and LH_n/A is essentially as described in Example 1 but with the following differences:

Materials

Lectin from *E. corallodendron* (EcL) was obtained from Sigma Ltd.

Figure 3 illustrates the purification scheme for the EcL-LH_n/A conjugate. Samples were applied to 4-20% polyacrylamide gradient gels and subjected to electrophoresis prior to staining with Coomassie blue. Lane 1 = molecular weight markers. Lane 2 represents the post-lactose affinity purified sample of EcL-LH_n/A. Lane 3 is a sample of pre-lactose affinity purified (size-exclusion chromatography only) EcL-LH_n/A. Lane 4 is a sample of pre-lactose affinity purified ExL-LH_n/A.

Example 3. The Production of a conjugate between a lectin from *Glycine max* and LH_n/A

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The procedure for production of a conjugate between a lectin from *Glycine max* and LH_n/A is essentially as described in Example 1 but with the following differences:

Materials

- 5 Lectin from *G. max* (SBA) was obtained from Sigma Ltd.

Method

For the affinity chromatography step an immobilised N-acetylgalactosamine (GalNAc) column was used and specific SBA-LH_n/A was eluted by the addition of 0.3M lactose.

- 10 Figure 4 illustrates SDS-PAGE profile changes during the purification scheme for SBA-LH_n/A. SBA-LH_n/A was purified from crude conjugate mixture by Superose-12 size-exclusion chromatography and immobilised N-acetylgalactosamine affinity chromatography. Samples were subjected to SDS-
15 PAGE on 4-20% polyacrylamide gels. Lanes 6-8 were run in the presence of 0.1M DTT. Lanes 1 (&7) and 2 (&8) indicate SBA and SPDP-derivatised LH_n/A respectively, prior to conjugation. Lanes 3, 4 & 5 (&6) represent conjugation mixture, post-Superose-12 and post-affinity chromatography
20 samples respectively. Lane 5 is therefore indicative of the profile of the final conjugate material. Molecular weight markers are represented in lanes Mr with sizes indicated on the figure.

- The absence of free lectin monomers was confirmed by
25 native non-denaturing PAGE analysis as illustrated in Figure 5. Samples were separated using 4-20% polyacrylamide gel for 6.75 hours, 125V, 4°C. The electrophoresis profile of SBA-LH_n/A (lane 1) was compared to those of SBA lectin only (lane 2) and LH_n/A (lane 3). A
30 range of marker proteins were analysed alongside; apoferritin (lane 6), β-amylase (lane 8), alcohol dehydrogenase (lane 7) and albumin (lane 9). Approximate molecular sizes are indicated.

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Example 4. Activity of ExL-LH_N/A in primary neuronal cultures

The dorsal root ganglia contain the cell bodies of primary nociceptive afferent neurons. It is well established that in primary *in vitro* cultures of this tissue the neurons retain many of the characteristics of the nociceptive afferent. These characteristics include the ability to release neuropeptides such as substance P in response to chemical stimuli known to cause pain *in vivo* (e.g. capsaicin). Neurons anatomically adjacent to those of the DRG include those of the spinal cord. Cultures of SC neurons prepared from embryonic rats can be established *in vitro* and the release of neurotransmitter (³H-glycine) under potassium stimulation can be assessed. As such, the eSC neurons represent a model cell for testing the selectivity of the agents described.

The selectivity of the ExL-LH_N/A agent for eDRG over eSC neurons is clearly illustrated in Figure 6. The dose curves document the effectiveness of ExL-LH_N/A in an *in vitro* cell culture model by comparing inhibition of neurotransmission in eDRG with eSC neurons.

Materials

Substance P enzyme linked immunosorbent assay kits were from Cayman Chemical Company.

Western blot reagents were obtained from Novex

Monoclonal antibody SMI-81 was from Sternberger Monoclonals Inc.

Methods

Primary cultures of dorsal root ganglion and embryonic spinal cord neurons were established following dissociation of the ganglia dissected from rat embryos (embryological age 12-15 days). For the preparation of

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eDRG neurons, the cells were plated into 12 well plates at an initial density of 3×10^5 cells/well in a medium containing NGF (100 ng/ml). After one day in culture, fresh medium containing cytosine arabinoside (10×10^{-6} M) was added to kill non-neuronal cells. After 2-4 days the cytosine arabinoside was removed. After several more days in culture the medium was replaced with fresh medium containing conjugate or LH_n.

For the preparation of eSC neurons, Cells were plated onto poly-D-lysine coated 12 well plates (Costar) at a density of 2×10^6 cells per well (1 ml/well). 'Plating' medium was MEM with Earles Salts (Sigma), containing 5% foetal bovine serum (FBS), 5% heat inactivated horse serum (HS), 0.6% dextrose, 1.5g/l NaHCO₃ and 2 mM L-glutamine. Cultures are incubated at 37°C with 10% CO₂. The medium was changed to 'feeding' medium (plating medium minus the FBS with N1 (Sigma) 1/50 supplement) after one day. When glial cells became almost confluent anti-mitotic agents (15 microgrammes /ml 5-fluoro-2'-deoxyuridine (FdU) and 35 microgrammes /ml uridine (U)) were added for a further 2-3 days. Cells were cultured for at least 3 weeks prior to use.

The cells were incubated with these agents for varying times and then tested for their ability to release the neurotransmitters glutamate and substance P (eDRG) or glycine (eSC). After the release assays were performed the cells were lysed and the hydrophobic proteins were extracted by phase partitioning with Triton-X-114 following the method outlined in Boyd, Duggan, Shone and Foster (J. Biol. Chem. 270, 18216-18218, 1995).

Substance P release assay

The release of endogenous substance P was effected by collecting cell supernatants after treating the cells for 5 min with either a physiological balanced salt solution

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or a balanced salt solution in which the potassium ion concentration had been raised to 100 mM with consequent reduction in the sodium ion concentration to maintain isotonicity. Total substance P was measured after
5 extraction in 2 M acetic acid, 0.1% trifluoroacetic acid and subsequent dehydration. Substance P immunoreactivity was measured using an enzyme immunoassay kit (Cayman Chemical Company).

[³H]Glutamate release assay

10 The release of glutamate was measured after loading the cells with [³H]glutamine as a radiotracer. The [³H]glutamine is converted to [³H]glutamate in the cell, and it is this [³H]glutamate that is taken up by synaptic vesicles and released upon depolarisation of the neuron.
15 The cells are loaded with the [³H]glutamine (5 X10⁻⁶ Ci/ml in HEPES-buffered MEM) for 2 h, then washed twice with HEPES-buffered MEM and thrice with balanced salt solution (BSS). Basal release was assessed with a 3 min incubation with BSS. Stimulated release was determined by a 3 min
20 incubation with BSS in which the potassium concentration had been elevated to 80-100 mM with a consequent reduction in the sodium concentration to maintain isotonicity. All manipulations were performed at 37°C. The cells were lysed by the addition of Triton-X-100 (0.1%, v/v). For the
25 basal and stimulated release superfusates the glutamate was separated from the glutamine by ion-exchange chromatography over Dowex-1 resin. The relevant fractions were analysed for ³H content by liquid scintillation counting.

30 *[³H] Glycine release assay*

The release of glycine was measured after loading the cells with [³H]glycine as a radiotracer. The [³H]glycine is taken up by synaptic vesicles and released upon depolarisation of the neuron. The cells are loaded with

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the [^3H]glycine (2×10^{-6} Ci/ml in HEPES-buffered MEM) for 2 h, then washed once with HEPES-buffered MEM and thrice with balanced salt solution (BSS). Basal release was assessed with a 5 min incubation with BSS. Stimulated
5 release was determined by a 5 min incubation with BSS in which the potassium concentration had been elevated to 56 mM with a consequent reduction in the sodium concentration to maintain isotonicity. All manipulations were performed at 37°C. The cells were lysed by the addition of 2 M acetic
10 acid, 0.1% trifluoroacetic acid. Fractions were analysed for their ^3H content by liquid scintillation counting and inhibition of release determined.

Figure 6 illustrates the activity of ExL-LH_N/A on release of neurotransmitter from eDRG and eSC neurons. Both eDRG
15 and eSC cultures were exposed to a range of ExL-LH_N/A concentrations (1 ml volumes) for three days. The percentage inhibition of eDRG substance P (n) and eSC [^3H]-glycine (?) release is in comparison to untreated controls. The data shown is representative of =3
20 determinations. IC₅₀ for eDRG was determined to be $3.66 \pm 0.92 \mu\text{g/ml}$. An inhibition of 50% was not obtained for eSC using the concentration range employed.

Western blotting

ExL-LH_N/A was applied to eDRG for 16 hours. After the
25 determination of neurotransmitter release the cells were lysed by the addition of 2 M acetic acid, 0.1% trifluoroacetic acid and subsequently dehydrated. To extract the membrane proteins from these mixtures Triton-X-114 (10%, v/v) was added and incubated at 4°C for 60 min,
30 the insoluble material was removed by centrifugation and the supernatants were then warmed to 37°C for 30 min. The resulting two phases were separated by centrifugation and the upper phase discarded. The proteins in the lower
35 phase were precipitated with chloroform/methanol for analysis by Western blotting.

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The extracted protein samples were applied to 4-20% polyacrylamide gradient gels and subjected to electrophoresis prior to transfer to nitrocellulose. Proteolysis of SNAP-25, a crucial component of the neurosecretory process and the substrate for the zinc-dependent endopeptidase activity of BoNT/A, was then detected by probing with an antibody (SMI-81) that recognises both the intact and cleaved forms of SNAP-25 (Figure 2). Proteins blotted onto nitrocellulose were probed with antibody SMI-81. Lanes 1-3, 4-6, 7-9 and 10-12 represent cells treated with medium, 40 microgrammes/ml ExL, 20 microgrammes/ml ExL and 40 microgrammes/ml LH_N/A respectively. Densitometric analysis of these data determined the %SNAP-25 cleavage to be 52.7% and 37.0% for 40 and 20 microgrammes/ml respectively.

Example 5. Activity of SBA-LH_N/A in primary neuronal cultures

Using methodology described in Example 4, the activity of SBA-LH_N/A in primary neuronal cultures was assessed. The selectivity of the SBA-LH_N/A conjugate for eDRG over eSC neurons is illustrated in Figure 7. Both eDRG and eSC cultures were exposed to a range of SBA-LH_N/A concentrations (1 ml volumes) for three days. The percentage inhibition of eDRG substance P (n) and eSC [³H]-glycine (O) release is in comparison to untreated controls. The data is the mean of three determinations \pm SE. The curves shown are representative of two experiments. IC₅₀ values for eDRG neurons were determined to be 1.84 and 7.6 microgrammes/ml. It is observed that SBA-LH_N/A exhibits a clear selectivity of the inhibition of neurotransmitter release from eDRG relative to eSC neurons. These data therefore confirm observations described for ExL-LH_N/A above and highlight the properties of galactose-specific lectins.

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Example 7. Activity of WGA-LH_n/A in primary neuronal cultures

Using methodology described in Example 4, the activity of WGA-LH_n/A in primary neuronal cultures was assessed. WGA represents an example of a non-galactosyl targeted lectin and therefore serves as an indicator of the properties of conjugate that do not recognise galactosyl moieties. The lack of selectivity of the WGA-LH_n/A conjugate for eDRG over eSC neurons is illustrated in Figure 8. eDRG and eSC neurons were exposed to a range of concentrations of WGA-LH_n/A for 3 days prior to assay of stimulated release of neurotransmitter (substance P and glycine respectively). Each conjugate concentration was assessed in triplicate and results are expressed as percentage inhibition compared to untreated controls. Panels A and B represent dose response curves from one experiment representative of ≥ 3 for eDRG and eSC neurons respectively. Each point shown is the mean of three determinations \pm SE of the mean. IC₅₀ data for the effects of WGA-LH_n/A was calculated to be 0.34 ± 0.06 microgrammes /ml (eDRG) and 0.06 ± 0.09 microgrammes /ml (eSC), indicating the lack of C-fibre selectivity.

Example 8. Activity of ExL-LH_n/A in an electrophysiological model of pain

A dose of 45 microgrammes of ExL-LH_n/A in a 10 microlitres volume of vehicle was given by intrathecal injection to rats between lumbar sections L4-L5, 24 hours prior to electrophysiological analysis of neuronal activity. Animals were allowed to recover and movement was not restricted prior to sacrifice and analysis. The results from a group of 3 animals with 10 neurons recorded per animal, show that there was a 73% reduction in the C-fibre responses of the neurones (Figure 9A) although the stimulus threshold is only slightly elevated (Figure 9B). Inhibition of C-fibre responses would lead to a decrease

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in the transmission of pain signals and these data are indicative of the analgesic effect of conjugate ExL-LH_N/A. There was also a significant decrease in the A_δ response (Figure 9C). These fibres are also implicated in the transmission of noxious stimuli and this result emphasises the analgesic effect of ExL-LH_N/A. A_δ neurons, a cell type that is not involved in transmission of noxious stimuli, were essentially unaltered in their responses to this stimulus (Figure 9D). The lack of affect on the A_δ-fibre neurons is indicative of the selectivity of ExL-LH_N/A for the neurons central to the transmission of pain signals.

Example 9. Activity of ExL-LH_N/A in behavioural models of pain

In an accepted *in vivo* model of pain, the mouse hotplate test, ExL-LH_N/A has been demonstrated to exhibit analgesic properties. Figure 10 illustrates the data obtained for ExL-LH_N/A where it is compared to a supramaximal dose of morphine. ExL-LH_N/A was applied intrathecally (30 microgrammes in a 5 microlitre vehicle volume) to each of a group of 10 mice and analgesic response in the hot plate test determined. Data is presented as hot plate latency (seconds) plotted against assay time (P = pre-treatment, 0-5 = hours post application). Onset of ExL-LH_N/A action had apparently reached a plateau at 1 hour that remained constant for at least 5 hours. The level of analgesia is similar to a supramaximal dose (50 microgrammes, 20X mouse EC₅₀) of morphine in this test, but is of much longer duration. This level of morphine achieves a maximal effect at 1 hour and then returns to control levels over a period of 5 hours. These data represent a clear indication of the analgesic potential of agents such as ExL-LH_N/A.

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Materials

Adult outbred mice (MF1) of either sex, weight range 20 to 30g.

Methods

- 5 Test material is injected into the intrathecal space of anaesthetised mice using a 30 gauge disposable needle attached to a 50 microlitre Hamilton syringe. The site of injection was normally chosen to be between lumbar vertebrae 5 and 6. The needle is inserted into the tissue
- 10 to one side of the vertebrae so that it slips into the groove between the spinous and transverse processes. The needle is then moved carefully forward to the
- 15 intervertebral space. 5 microlitres of test material is then injected into the intrathecal space and the needle withdrawn. The skin incision is then closed with a single wound clip and the animal placed in a box to allow recovery.

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Claims

1. An agent for the treatment of pain that comprises a galactose-binding lectin linked to a derivative of a clostridial neurotoxin, in which the derivative of the clostridial neurotoxin comprises the L-chain, or
5 a fragment thereof, which includes the active proteolytic enzyme domain of the light (L) chain, linked to a molecule or domain with membrane translocating activity.
- 10 2. An agent according to Claim 1 in which the membrane translocation domain is derived from the heavy chain of a clostridial toxin.
3. An agent according to Claim 1 in which the membrane translocation domain is derived from a non-
15 clostridial source.
4. An agent according to any preceding Claim in which the lectin binds to oligosaccharides that contain terminal β -D-galactosyl residues
- 20 5. An agent according to any preceding Claim in which the lectin binds to oligosaccharides that contain terminal α -D-galactosyl residues
6. An agent according to any preceding Claim in which the lectin binds to oligosaccharides that contain N-acetylgalactosamine
- 25 7. An agent according to any previous Claim in which the lectin is derived from a species of plant.

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8. An agent according to the previous Claim in which the lectin is derived from a species of the genus *Erythrina*.
- 5 9. An agent according to Claim 8 in which the lectin is derived from *E. cristagalli*.
- 10 10. An agent according to Claim 8 in which the lectin is derived from *E. corallodendron*.
11. An agent according to Claims 7 in which the lectin is obtained from *Glycine max*.
- 10 12. An agent according to Claims 7 in which the lectin is obtained from *Arachis hypogaea*.
13. An agent according to Claims 7 in which the lectin is obtained from *Bandeirea simplicifolia*.
- 15 14. An agent according to Claim 1-6 in which the lectin is of mammalian origin.
15. An agent according to Claim 1-6 in which the lectin is obtained from bacteria.
16. An agent according to Claim 15 in which the lectin is obtained from *Pseudomonas aeruginosa*.
- 20 17. An agent according to any preceding Claim in which the lectin has been produced using recombinant technology.

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18. An agent according to any preceding Claim in which the lectin has been enzymatically modified.
19. An agent according to any preceding Claim in which the lectin has been chemically modified.
- 5 20. An agent according to any preceding Claim which comprises the lectin coupled to a clostridial neurotoxin in which the H_c domain of the H-chain is removed or modified.
- 10 21. An agent according to any preceding Claim in which the H-chain is modified by chemical derivatisation to reduce or remove its native binding affinity for motor neurons.
- 15 22. An agent according to any of Claims 1-20 in which the H-chain is modified by mutation to reduce or remove its native binding affinity for motor neurons.
23. An agent according to any of Claims 1-20 in which the H-chain is modified by proteolysis.
- 20 24. An agent according to Claim 20 in which the H_c domain is completely removed leaving only the H_N-fragment of a clostridial neurotoxin.
25. An agent according to any preceding Claim in which the clostridial neurotoxin component is obtained from botulinum neurotoxin.

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26. An agent according to any preceding Claim in which the clostridial neurotoxin component is obtained from botulinum neurotoxin type A.
- 5 27. An agent according to Claims 1-25 in which the clostridial neurotoxin component is obtained from botulinum neurotoxin type B.
28. An agent according to any of Claims 1-25 which is formed by the coupling of a galactose-binding lectin to the LH_n fragment of botulinum neurotoxin type A.
- 10 29. An agent according to Claim 28 which is formed by the coupling of the galactose-binding lectin from *Erythrina cristagalli* to the LH_n fragment of botulinum neurotoxin type A.
- 15 30. An agent according to Claim 28 which is formed by the coupling of the galactose-binding lectin from *Erythrina corallodendron* to the LH_n fragment of botulinum neurotoxin type A.
- 20 31. An agent according to Claim 28 which is formed by the coupling of the galactose-binding lectin from *Glycine max* to the LH_n fragment of botulinum neurotoxin type A.
- 25 32. An agent according to any preceding Claim in which the H-chain is obtained from a different clostridial neurotoxin than that from which the L-chain is obtained.

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33. An agent according to Claim 32 in which the H-chain is obtained from botulinum neurotoxin type A and the L-chain from botulinum neurotoxin type B.
- 5 34. An agent according to Claim 32 in which the H-chain is obtained from botulinum neurotoxin type A and the L-chain from tetanus neurotoxin.
35. An agent according to Claims 33 and 34 in which the H-chain component is the H_w fragment of botulinum neurotoxin type A.
- 10 36. An agent according to any preceding Claim in which the L-chain or L-chain fragment is linked to the H-chain by a direct covalent linkage.
- 15 37. An agent according to any of Claims 1-35 in which the L-chain or L-chain fragment is linked to the H-chain by a covalent linkage which includes one or more spacer regions.
38. An agent according to any preceding Claim in which the clostridial neurotoxin derivative incorporates polypeptides produced by recombinant technology.
- 20 39. An agent according to any preceding Claim in which the lectin is linked to the clostridial neurotoxin-derived component by a direct covalent linkage.
- 25 40. An agent according to any of Claims 1-38 in which the lectin is linked to the clostridial neurotoxin-derived component by a covalent linkage which includes one or more spacer regions.

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41. An agent according to any preceding Claim in which the lectin and clostridial neurotoxin components are produced as a recombinant fusion protein.
- 5 42. An agent according to any preceding Claim in which the lectin protein has been modified from its native polypeptide sequence whilst retaining an ability for the protein to bind to oligosaccharide structures, in which the terminal residue is derived from galactose or N-acetylgalactosamine.
- 10 43. An agent according to Claim 42 in which the protein modification results from modification of the nucleic acid coding for the lectin protein from its native sequence.
- 15 44. An agent according to any preceding Claim which prevents the release of a neurotransmitter or neuromodulator from a primary sensory afferent.
45. An agent according to any preceding Claim which inhibits the release of a neurotransmitter or neuromodulator from a primary nociceptive afferent.
- 20 46. A method for obtaining an agent according to any preceding Claim which comprises the covalent attachment of a galactose-binding lectin to a derivative of a clostridial neurotoxin, in which the derivative of the clostridial neurotoxin comprises
25 the L-chain or an L-chain fragment which includes the active proteolytic enzyme domain of the light (L) chain, linked to a molecule or domain with membrane translocating activity.

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47. A method for obtaining an agent according to any of Claims 1-58 which comprises the covalent attachment of a galactose-binding lectin to a derivative of a clostridial neurotoxin with the inclusion of one or more spacer regions, in which the derivative of the clostridial neurotoxin comprises the L-chain or an L-chain fragment which includes the active proteolytic enzyme domain of the light (L) chain, linked to a molecule or domain with membrane translocating activity.
48. An method according to Claim 47 in which the membrane translocation domain is derived from the heavy chain of a clostridial toxin.
49. An method according to Claim 47 in which the membrane translocation domain is derived from a non-clostridial source.
50. A method for obtaining an agent according to any of Claims 1-45 which comprises constructing a genetic construct which codes for the agent, incorporating said construct into a host organism and expressing the construct to produce the agent.
51. A method of controlling the release of a neurotransmitter or neuromodulator from a primary sensory afferent by applying the agent of any one of Claims 1-45.
52. A method of controlling the release of a neurotransmitter or neuromodulator from a primary nociceptive afferent by applying the agent of any one of Claims 1-45.

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53. A method of controlling the transmission of sensory information from a primary sensory afferent to a projection neuron by applying the agent of any one of Claims 1-45.
- 5 54. A method of controlling the transmission of sensory information from a primary nociceptive afferent to a projection neuron by applying the agent of any one of Claims 1-45.
- 10 55. A method of controlling the sensation of pain by applying the agent of any one of Claims 1-45.
56. Use of the agent according to any one of Claims 1-45 or a pharmaceutically acceptable salt thereof as a medicament for the alleviation of pain.
- 15 57. Use of the agent according to any one of Claims 1-45 or a pharmaceutically acceptable salt thereof as a medicament for the prevention of pain.
58. Use of the agent according to any one of Claims 1-45 in the manufacture of a medicament for the alleviation of pain.
- 20 59. Use of the agent according to any one of Claims 1-45 in the manufacture of a medicament for the prevention of pain.
- 25 60. A method of alleviating pain which comprises administering an effective dose of the agent according to any one of Claims 1-45.

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61. A method of preventing pain which comprises administering an effective dose of the agent according to any one of Claims 1-45.

Figure 1

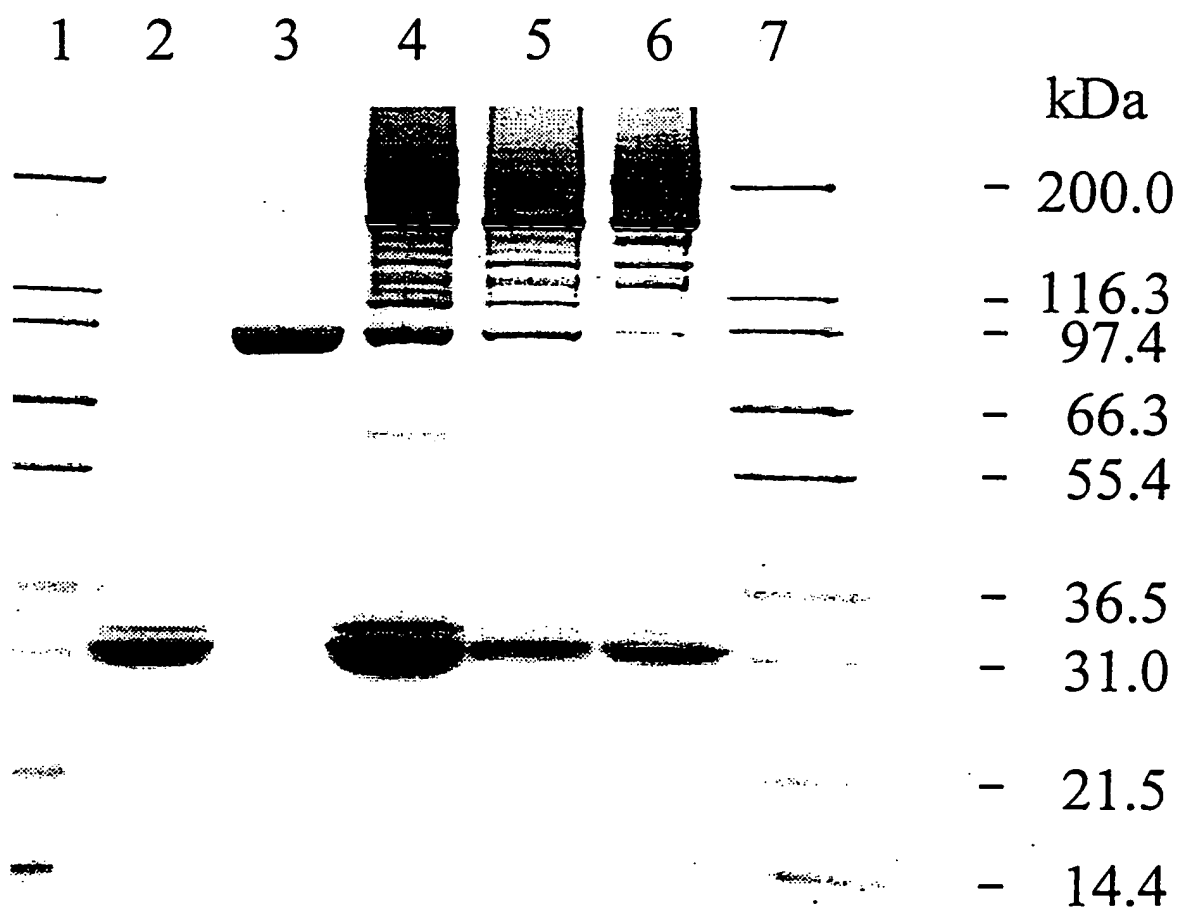


Figure 2

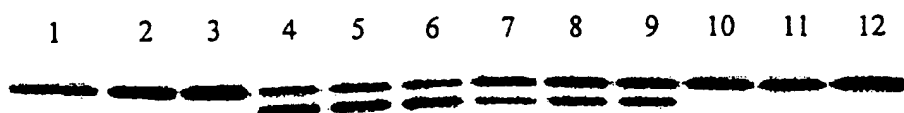
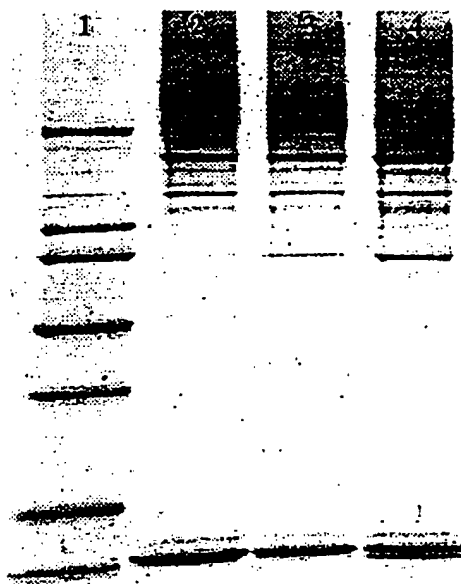


Figure 3



Figur 4

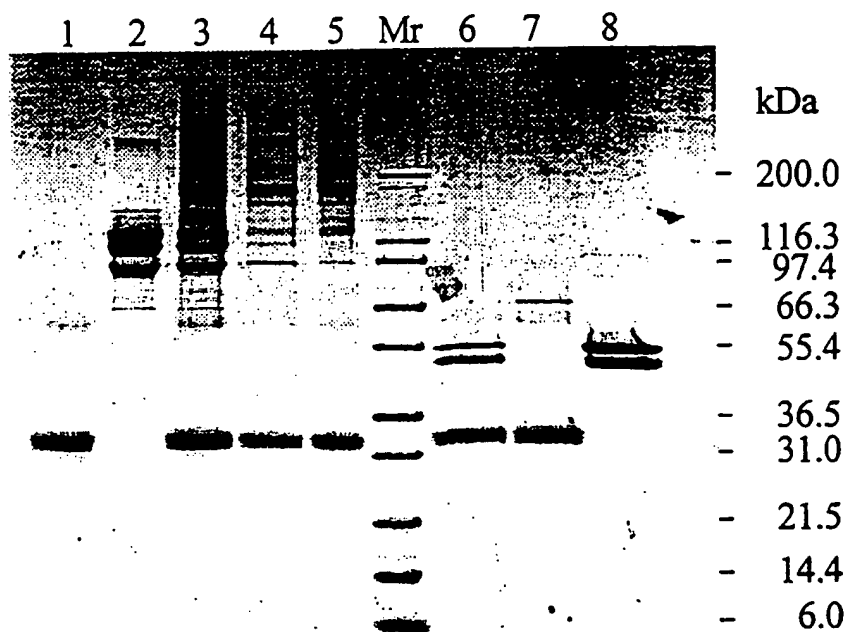
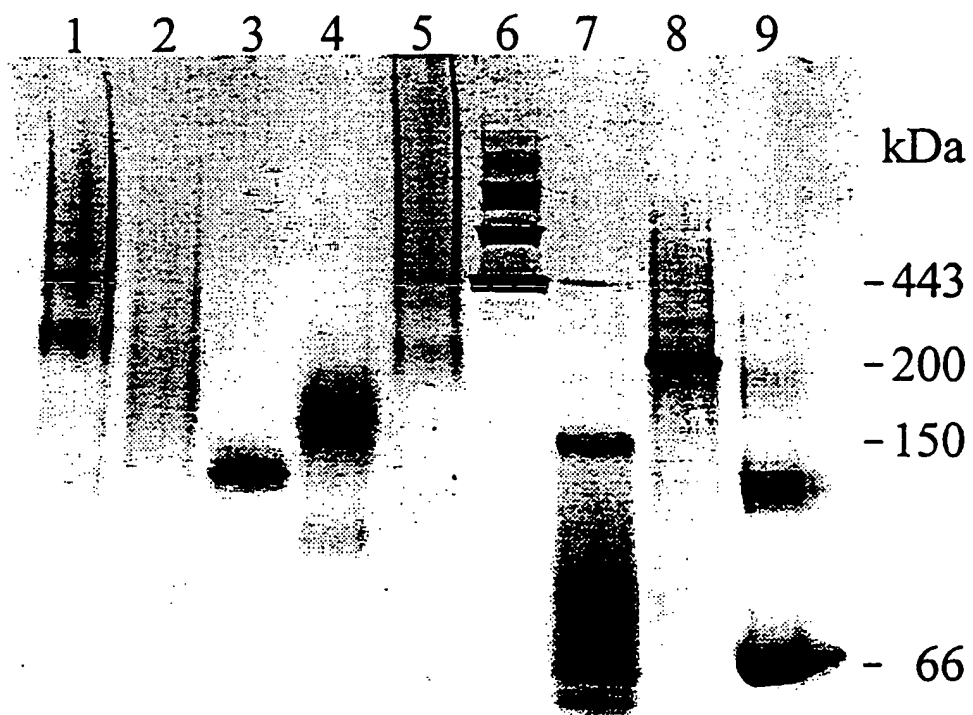


Figure 5



Figur 6

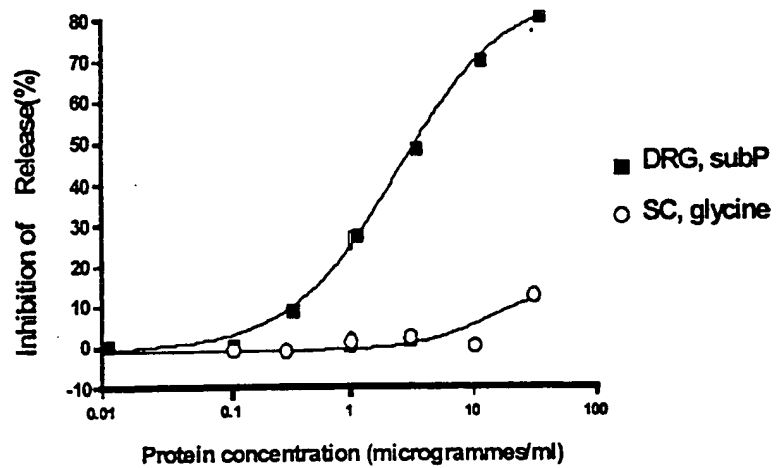


Figure 7

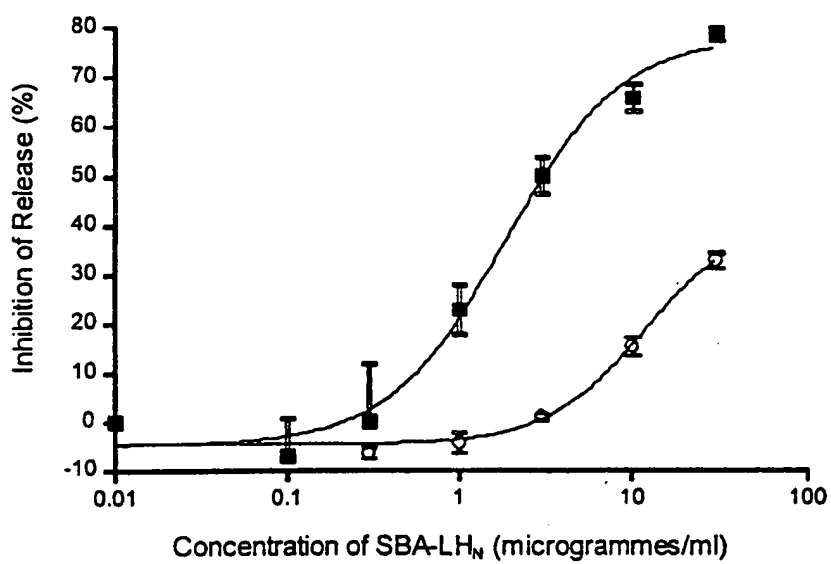
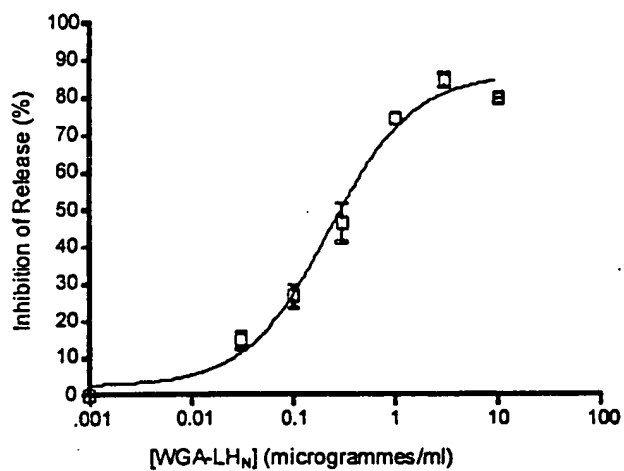


Figure 8

Panel A: eDRG



Panel B: eSC neurons

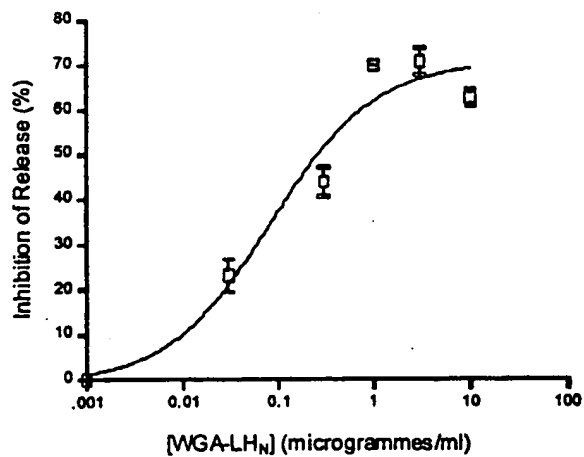
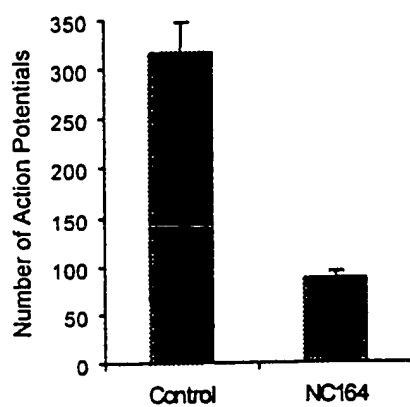
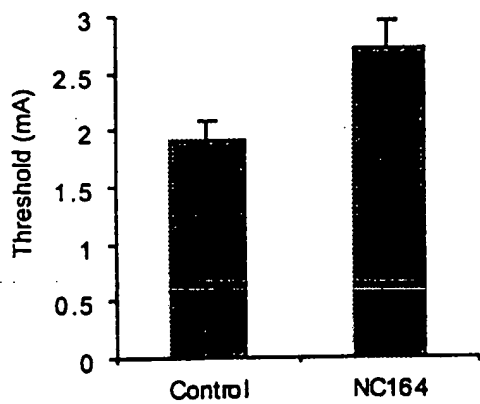


Figure 9

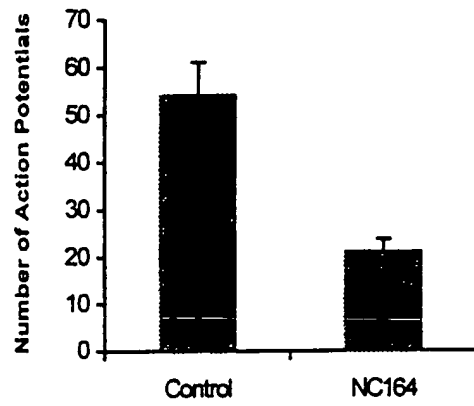
A.



B.



C.



D.

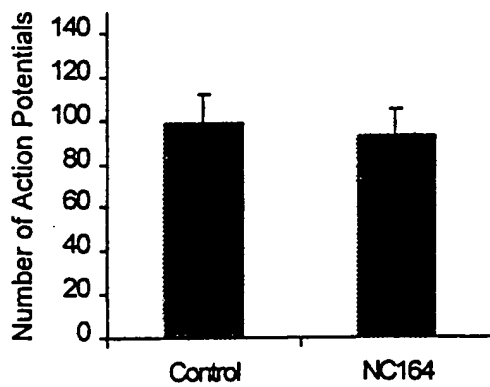
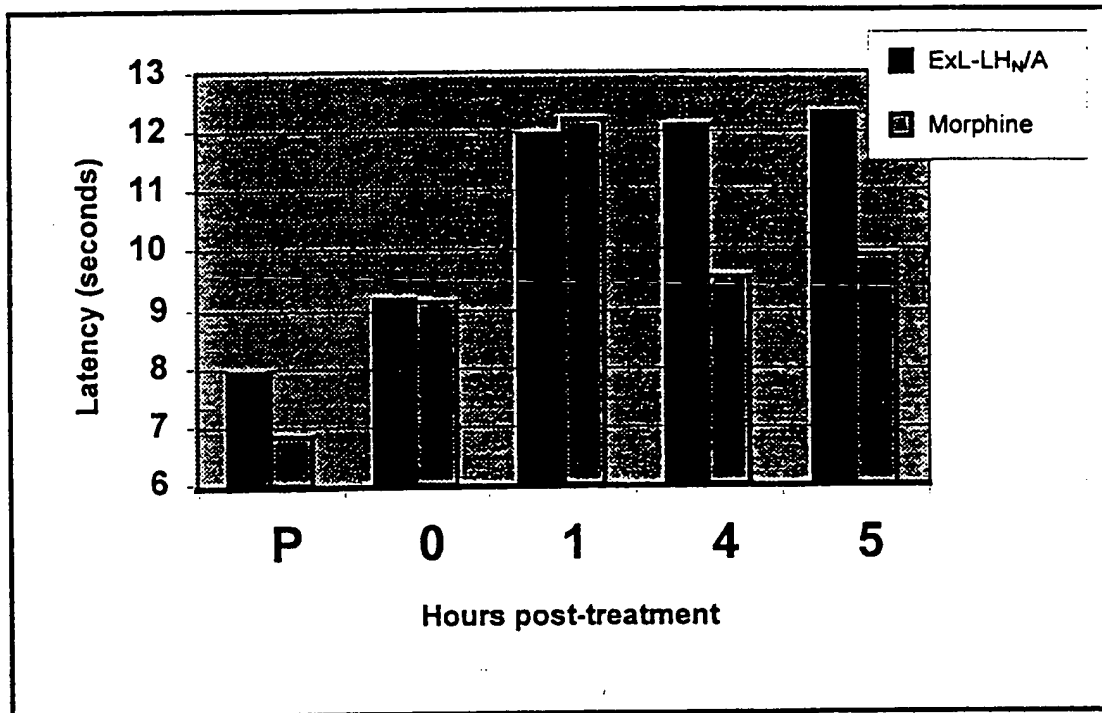


Figure 10



INTERNATIONAL SEARCH REPORT

Int. l. Application No

PCT/GB 98/03001

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 33273 A (SPEYWOOD LAB LIMITED ;MICROBIOLOGICAL RES AUTHORITY (GB); FOSTER K) 24 October 1996 cited in the application	1-61
Y	see page 12, line 1 - page 13, line 8; claims see page 13, line 9 - line 13	1-61
P, X	WO 98 07864 A (FOSTER KEITH ALAN ;MICROBIOLOGICAL RES AUTHORITY (GB); QUINN CONRA) 26 February 1998 see claims	1
A	WO 94 28923 A (ALLERGAN INC) 22 December 1994 see claims	
	--- -/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

26 January 1999

Date of mailing of the international search report

09/02/1999

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

Berte, M

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/03001

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 18790 A (UNIV MONTANA RES DEV INST ;PASCUAL DAVID (US); BOND CLIFFORD (US);) 29 May 1997 see claims 1,4,5,9,29,30 -----	1-61
A	WO 95 32738 A (ALLERGAN INC ;DOLLY JAMES OLIVER (GB); AOKI KEI ROGER (US); WHEELER) 7 December 1995 -----	
Y	STREIT, WOLFGANG J. ET AL: "Histochemical localization of galactose-containing glycoconjugates in sensory neurons and their processes in the central and peripheral nervous system of the rat" J. HISTOCHEM. CYTOCHEM. (1985), 33(10), 1042-52 CODEN: JHCYAS;ISSN: 0022-1554, 1985, XP002091075 see abstract -----	1-61

INTERNATIONAL SEARCH REPORT

...ernational application No.

PCT/GB 98/ 03001

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 51-55,60-61
is(are) directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

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